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(54) Title: FINGERPRINTING OF NUCLEIC ACIDS, PRODUCTS AND METHODS

(57) Abstract

Improvements to nucleic acid fingerprinting are disclosed. The method generates profiles of increased fidelity charactristic of the nucleic acids analyzed. Novel primers and other means are taught. Useful products are disclosed.

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TITLE OF THE INVENTION

FINGERPRINTING OF NUCLEIC ACIDS, PRODUCTS AND METHODS

FIELD OF THE INVENTION

The invention relates to nucleic acid "fingerprinting of nucleic acids".

RELATED PATENT APPLICATIONS

This patent application is a continuation-in-part of pending patent application Serial No. 08/006,380, filed January 19, 1993 entitled "DNA Amplification Fingerprinting", inventors, Gustavo Caetano-Anolles, Brant Bassam and Peter Gresshoff.

That application "the first parent" application is incorporated herein by reference. The method has been nick-named "DAF". It provides the highest resolution of fingerprint product known to date.

This patent application is also a continuation-in-part of Serial No. 07/676,869, "the second parent" application, filed March 28, 1991, entitled "DNA Silver Staining", inventors, Gustavo Caetano-Anolles, Brant Bassam and Peter Gresshoff, which is also incorporated herein by reference.

BACKGROUND OF THE INVENTION

A novel method for DNA fingerprinting that uses at least one oligonucleotide to prime arbitrary segments of a DNA template to produce a characteristic set of amplified fragments is being shown to be of increasing value in the analysis of genetic relationships. Fingerprint complexity varies from very simple, and thus ideal for genome mapping, to highly complex and more suitable for fingerprinting. See Bio/Technology, Vol. 10: 937, September 1992 incorporated herein by reference and attached (Exhibit 1).

DNA amplification fingerprinting (DAF) is the enzymatic amplification of arbitrary stretches of DNA which is directed by short oligonucleotide primers of arbitrary sequence to generate complex but characteristic DNA fingerprints.

The restriction mechanism proposed by the inventors can be seen in Amplifying DNA with Arbitrary Oligonucleotide Primers, Review 1993, Cold Spring Harbor Laboratory Press which is incorporated herein by reference and attached hereto (Exhibit 2). Of particular interest is the description of the step by step amplification described at pages 2 and 3.

Terms and terminology used in conjunction with the invention are known in the art. For instance, "oligonucleotide", "primer", "restriction endonuclease" and "restriction enzymes",

"DNA polymorphism", "Restriction fragment, length polymorphism", ("RFLP"), "random nucleic acid fragment", "DNA fingerprinting", or "DNA typing", "genotyping", "profiling", "DNA identification analysis", or "DNA polymorphism", "polymerase chain reaction" ("PCR"), "DNA amplification", "random amplified polymorphic DNA" ("RAPD"), "amplicons" and "DNA amplification fingerprinting" ("DAF") are discussed in the patent and other scientific literature, such as in the U.S. Patent Nos. 4,683,202 (Mullis), 5,126,239 (Livak et al.), PCT Publication No. WO 92/03567 Caetano-Anolles et al., which are incorporated herein by reference. For "Genomes" ("complex and simpler", see Genes IV by Benjamin Lewin, Chapter 24 (1990), ranging from as little as 10^6 for a mycoplasma to as much as 10^{11} bp for some plants and amphibians, which is incorporated herein by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows photographs of gels of profiles obtained using hairpin primers HP_7 and different primer sequences.

Figure 2 shows photographs of gels of profiles using typical hairpin primers with different hairpins.

Figure 3 shows photographs of gels in which the primers have certain bases substituted as illustrated by I and/or by N, N representing any one of the four bases.

SUMMARY OF THE INVENTION

The present invention provides novel improvements in methods and products in DNA amplification fingerprinting (DAF). Products of the invention provide improvements not only in DAF, but also in multiple arbitrary amplicon profiling (MAAP) techniques, like in random amplified polymorphic DNA (RAPD) analysis or modifications thereof.

The invention provides a method of reamplifying nucleic acid product(s) of DAF to synthesize nucleic acid sequences extended at the 5' end by functional regions.

Another embodiment of the invention provides novel oligonucleotide primers, particularly short primers, with a 3' end of a defined number of nucleotides and their use in DAF or in other MAAP techniques.

An important embodiment of the invention provides various techniques and means to decrease amplification from mismatched primers and to optimize product formation resultant from the functions of the 3' end of the primer.

In another embodiment, the invention provides novel arbitrary oligonucleotide primers which include at the 5' end, a hairpin structure or other equivalent structure and their use in DAF or in other MAAP techniques.

In another somewhat related embodiment, the invention

provides novel arbitrary oligonucleotide primers which comprises purine or pyrimidine substituted polyamide (PNA, for polyamide nucleic acid) and their use in DAF or in other MAAP techniques.

The invention further provides multiple endonuclease digestion of selected template DNA followed by the treatment of the DNA digestion by DAF or other MAAP techniques.

The invention provides the use of improved highly thermo-stable DNA polymerases, truncated derivatives of <u>Thermo</u> <u>Aquaticus</u> (AmpliTaq), which are especially well suited for use in DAF and other MAAP techniques.

In accordance with the invention, there are provided improved nucleic acid amplification parameters for DAF such as cycling conditions, e.g. temperature, amounts of primer relative to nucleic acid template, the concurrent use of multiple primers, and other aspects that will become apparent from the more detailed description of the invention.

The various methods and products of the invention provide remarkable increase in information content (i.e. number of bands and polymorphisms) of fingerprints generated with the selected primer(s).

In accordance with the invention it should be noted that the various improvements in methodology and new products herein disclosed can be used individually in conjunction with what is called the DAF method, in general, the MAAP methodologies, or in

any combination thereof. For instance, the important feature of the invention as used hereinafter of using an arbitrary mini-hairpin oligonucleotide primer for nucleic acid amplification fingerprinting with only 3 terminal (3' end) single stranded nucleotides may be applied to any of the existing MAAP techniques like DAF or others which will be hereinafter described. The same observation applies to other embodiments disclosed herein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

A method of the invention comprises amplifying nucleic acid fragment(s) obtained from DAF with at least one arbitrary primer which has a common sequence with and which may be shorter, but preferably is longer than the primer used in the first amplification. For reamplification with the primer of selected length, a mixture of nucleic acid fragments or a single isolated nucleic acid fragment, preferably purified product obtained from a first amplification, may be used as the template.

A method of reamplification is further described in Mol. Gen. Genet. 235:157-165 (1992) ("MGG 1992") which is incorporated herein by reference be made a part of this disclosure and attached hereto as Exhibit 3. In Figures 3A-C it will be seen that primers of different lengths sharing a common sequence are shown amplifying a single isolated and purified product or a mixture of products

obtained from a first amplification with octamers.

Figures 5A, B, show the reamplification of all products of a single isolated and purified product obtained from using the shown octamer. Conversely, a pentamer and an octamer were used to reamplify the products originally generated by primers (Fig. 6A, B).

The reamplification fragments comprise at their respective 5' end an additional sequence which causes a shift in the visualization bands, which provides additional information about the nucleic acids analyzed.

Of great practical interest are such reamplification products which comprise selective tailored extended 5' end sequences for selected uses, for instance as hybridization probes for diagnostic use in targeting a gene and other uses. The products should be distinguished from DNA fragments with ligated ends of desired sequences.

Primers longer than 8 nucleotides, like 12 and 15 nucleotides, which produce 5' overhang on primer-template duplexes during annealing with the original template sequence, amplified template DNA in all cases. Larger primers, e.g. 30 nucleotides in length may be considered depending on the use intended for the products. Preferably, it is primers of at least 8 nucleotides in length which are extended as described herein.

The invention provides a further important embodiment

which relates to primers. The prior art generally teaches that the minimum useful primer length is an oligonucleotide of 9 bases (Williams et al. (1990), the RAPD method). The first parent application to the present application proposes at least 5 nucleotides for successful amplification and product visualization. It has now been discovered that in any length oligonucleotide primer it is the first 5 nucleotides from the 3' end which define what is called here the "core" region. Without these 5 nucleotides, no priming event is likely to take place. The next region comprising from nucleotide 6 to 8 hereby defined as the "optimizer" region, contributes to more efficient amplifications. It is the region which plays an important role in recognizing the priming target site. The following basic domain from 9 upward, for instance to 30, is considered a "5' extension tail" region. These additional nucleotides will alter the amplified spectrum only moderately. In accordance with the inventors' view, the primers thus can be considered as comprising starting from the 3' end, a core of 5 nucleotides followed by a domain "the optimizer" to a length of primers to a total of 7 or 8 nucleotides. For amplification of genomes of greater complexicity like soybean and human, preferably the primer will contain 8 nucleotides and it will contain 7 nucleotides for simpler genomes, like for bacteria and fungi. Addition of nucleotides to the 7 or 8 nucleotide domain, respectively, does not increase the information content of DNA

patterns significantly. Thus, in accordance with the invention, the core and the optimizer domains are the major determinants of the amplification reaction, conditioning the number and nature of amplification products produced. However, as taught hereinafter, it has been unexpectedly discovered in accordance with the invention that the core sequence can be reduced to 3 nucleotides.

These observations were based on studies where sets of related oligonucleotides where designed by removing nucleotides from the 5' end of an arbitrarily chosen sequence then used to direct amplification of several templates. Patterns generated by related primers of 5 to 8 nt in length were different in complexity and band distribution. Surprisingly, increasing primer length increased the number of amplification products. In contrast, primers of 8 and 10 nt in length produced virtually identical patterns, while patterns generated with longer primers were again divergent but showed some common bands. Base substitutions in the last two 5'-terminal nucleotides produced several variant amplification fragments, but the overall patterns remained very similar. This (as noted above) indicates that sequences beyond the 8 nt 3'-terminal region affect amplification only moderately.

These and related studies suggest that longer primers such as those used by other known methods may not be required. Such methods are in effect using the first 5-8 nucleotides of the 3' end of the primer effective domain. For further description of

these aspects of the invention, reference is made to Amplifying DNA with Arbitrary Oligonucleotide Primers, Gustavo Caetano-Anolles, cited above.

Although in the first parent case, the use of pentamers was taught, the full significance of the role of the various component domains of longer primers was not fully appreciated.

In connection with primers, it is noted that U.S. Patent No. 5,126,239 to Livak et al. teaches that it is preferable that the primer contain sequences that do not form a "hairpin" configuration. In accordance with the present invention, it has been found most unexpectedly that what appeared heretofore to be close to the functional limit for priming DNA amplification (5 oligonucleotides) is reducible even further to 3 nucleotides. In accordance with the invention, these primers comprise at the 3' end, 3 nucleotides and a hairpin at the 5' end. These primers are called herein "hairpin or mini-hairpin primers". These arbitrary primers, unlike other primers used heretofore in fingerprinting unexpectedly need only a minimum of a combination of any three nucleotides at the 3' end to amplify arbitrary stretches of nucleic acids from a nucleic acid template.

Figure 1 shows the use of sequence related primers having at the 5' end a mini-hairpin (HP_7) of sequence GCGAGC for the amplification of DNA.

Panel A shows amplification of DNA from indonesian fruit

bat (*Pteropus Hypomelanus*) soybean (*Glycine max* cv. Bragg) (Panel B) bacterium (E. coli strain Smith92) (Panel C), and bacteriophage (Lambda cI857ind1Sam7) (Panel D).

This and related work shows that a mini-hairpin primer with only a 3 base pair arbitrary domain (in the case, illustrated, CTG) produce reproducible fingerprints. It is to be noted that the hairpin sequence HP_7 *per se* is unable to produce any fingerprints, indicating that the most active annealing sequence is that of the 3' end.

It is not to be totally excluded that under other experimental conditions a primer with a domain with less than 3 base pairs could adequately produce reproducible fingerprints.

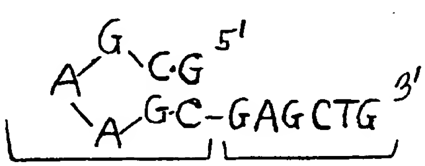
While it is not intended to be limited by this theory, it appears that amplification products initiated by a single primer share the particular characteristic of having a region of terminal hairpin symmetry at least as long as the primer itself. For efficient amplification of these products to occur, the primer should displace these hairpin loop complexes long enough for the DNA polymerase to anchor and stabilize the duplex by strand extension. The extent of hairpin loop interference will be variable for each fragment, allowing some fragments to be preferentially amplified. Studies in relation to this aspect of amplification by primers of increasing length suggests that when the primer is longer than the 8-nucleotide domain, it is better

able to compete for annealing with hairpin loops. This may be the reason why conventional fingerprinting methodology teaches using primers greater than 8 nucleotides in length. See Williams et al. 1991. Unexpectedly, it has been discovered, however that it is only necessary for the arbitrary mini-hairpin oligonucleotide primers to have at the 3' end, 3 nucleotides which basically determine amplification. It may be that the hairpin primers of the invention inhibit hairpin formation on the amplification products and make more priming sites accessible than heretofore available.

A most interesting consequence of this finding in accordance with the invention is that the invention now provides a close set of 64 different primers, which is the total of all combinations in a sequence of nucleotides containing any of the known 4 bases of DNA.

Accordingly, the invention provides a kit for amplification which comprises 64 different primers of 3 nucleotides of arbitrary sequence at its 3' end and a hairpin loop constituting the 5' end.

An illustrative mini-hairpin primer can be represented as follows:



where the 3' region is constituted of 4 nucleotides GCTH3', and the hairpin is constituted of a stem of 2 nucleotides of complementary bases and a single stranded loop of 3 nucleotides. Another primer was constructed with the same hairpin and CTG at the 3' end. Other illustrative primers are HP_7-CTG, HP_7-GCTG, HP_3-GAGCTG, HP_7-CCGAGCTG. See Figure 1 which shows the use of sequence related primers having at the 5' end a mini-hairpin HP_7 of sequence GCGAAGC for the amplification of DNA. Other primers synthesized included HP_7 = GCGAAGC, HP_8 = GCGAAAGC and HP_8 = GCGTTAGC. Figure 2 shows other mini-hairpins when amplifying soybean cv. Bragg or E. coli (isolated Smith 92), panel A and B, respectively. The mini-hairpins have the following sequence: HP_8-GCTG, HP_7-GCTG, HP_8-GAGCTG, HP_8-GAGCTG, HP_7-GAGCTG.

Many sequence variants for the hairpin loop of the 5' end of the primer form stable mini-hairpins in a similar manner as the HP_7. For instance, DNA fragment, d(GCGAAAGC) forms a stable hairpin structure consisting of a GAAA loop and only 2 GC pairs at the stem. It is within the scope of the invention that the mini-hairpin primers have different loop sequences and/or different stem sequences. To be considered are the following: d(CGGAAAGC), d(GCGNAAGC) where N is either A (shown), G, C, or T. The 3' end is constituted by any combination of 3 or more nucleotides.

Further, RNA fragments like r(GCGAAAGC) also form a hairpin loop on the 5' end of a selected primer.

It appears that preferably the arbitrary mini-hairpin oligonucleotide primers for use in the invention have more than 2 G-C base pairs as the stem region and contain 3 or 4 nucleotides in the loop region, including at least one A, preferred being either GAA or GA.

The arbitrary mini-hairpin oligonucleotide primers are very useful in conjunction with DAF, but also in any other multiple arbitrary amplicon profiling (MAAP) technique.

DNA amplification fingerprinting using arbitrary mini-hairpin oligonucleotide primers is carried out as follows. A modified primer was used to amplify arbitrary stretches of DNA from a DNA template using primers of related sequences as shown in Figure 1, having at the 5' end a mini-hairpin (HP_7) of sequence GCGAAAGC. The method used was as follows: DAF reactions were done in a total volume of 20-25 μl containing 3 μM primer, 0.3 units/μl AmpliTaq Stoffel DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), 200 μM of each deoxynucleoside triphosphates, 4 mM $MgSO_4$, 10 mM KCl, 4 mM $(NH_4)_2SO_4$, 0.1% Triton, 20 mM Tris-HCl (pH 8.3), and about 0.1 ng/μl of template DNA. The mixture was amplified in 35 cycles of 30 s at 96°C, 30 s at 30°C, and 30 s at 72°C in a recirculating hot-air thermocycler (Bios, New Haven, CT). Amplification products were separated in polyester-backed 5% polyacrylamide-urea minigels and stained with silver as described in Caetano-Anolles et al. Biotechnology Vol. 9:553-557, (1991) incorporated herein by

reference and attached (Exhibit 4). Wells were loaded with 3 μl of a 1/10 dilution of each amplification reaction mixed with 3 μl of loading buffer (5M urea and 0.02% xylene cyanol FF) and run at 100 V for about 80 min. Detection of DNA at the picogram level was by silver staining as described in Bassam et al. Anal. Biochem., Vol. 196, 80-83, (1991) as incorporated herein by reference and attached (Exhibit 5).

Using a mini-hairpin primer having only 3 nucleotides of arbitrary sequence gives a more reliable fingerprinting of small genomes as illustrated in Panels C and D of Figure 1, respectively an E. coli strain and bacterial phage Lambda.

A close set of primers representing the 64 combinations of these 3' terminal nucleotides each having a 5' HP_7 is made available by following the procedure outlined above. Similar fingerprinting with bacterial phage Lambda is obtainable.

When it is desired to use more than 1 primer, 2 or more of the primers of the kit can be used.

In accordance with the invention, further new primers have been constructed in which one or more oligonucleotides at the 3' or the 5' terminus of the arbitrary primer have been substituted by a selected base. Illustrative bases are hypoxanthine (I), 6H,8H03,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (P) and 2-amino-6-methoxyaminopurine (K). Other bases may be considered. Likewise, any of the four bases, A, G,C, T can be replaced by

another base. See Figure where 3 I represents inosine and N any one of the four bases: A, G, C, T.

From these studies, it appears that by the replacement of a nucleotide in such short primers, its discriminating function with respect to the priming site is at least in part hindered. The effective priming region has effectively become shorter and lost some of its specificity. This may have important applications not yet fully understood or explored.

In Figure 3, it will be seen that fingerprints can also be tailored by having these substitutions. In some cases profiles simplify considerably (especially when I is introduced at the 3' end) in other the profiles become too complicated (by introducing degenerate bases at the 3' end).

The results presented herein above show that a primer for MAAP techniques need not contain more than 3 nucleotides at the 3' end which "screen" the target DNA possible sites and anneals to them and that the region which was hereinabove described as the "5' extension tail" is not essential when it is replaced in part or totally by an appropriate optimizer. An illustration provided by the invention is the hairpin primer. However, the concept of modifying the 5' domain of a primer is not limited to a hairpin modification. Any other modification which has an equivalent effect is contemplated to be within the scope of the invention. Chemical modification of the 5' end may be performed.

It is possible to fluorophore label the DNA 5' end of the primer with FAM, blue, JOE, green, TAMRA, yellow, or ROX, red. Efficient amplification takes place. An illustration is the primer GTGACGTAGG fluorescently labelled at its 5' end with FAM which was used to amplify two different turfgrass DNA samples. Amplification products can be separated using a Gene Scanner ABI 362. See, Applications of RAPD Technology to Plant Breeding, Joint Plant Breeding Symposia Series, November 1, 1992, which is incorporated here by reference (Exhibit 6), and attached hereto. At least one primer may be biotinylated, i.e. covalently linked to biotin or an analog of biotin (biotin ovidin). Other groups designated as "reporter" groups can be used at the 5' end.

Another type of primer are those in which the 5' end is modified by a polyamide nucleic acid (PNA). Such polyamide is designed by replacing the deoxyribosephosphate backbone of DNA and with a achiralpolyamide backbone. Such oligomers recognize their complimentary target in double stranded DNA by strand displacement.

Accordingly, such primers comprise a 5' end of a DNA-PNA hybrid with a 3' end of 3 nucleotides (or more if desired), the DNA fragment constituting the balance of the primer such as 2 to 5 or 6 nucleotides, if desired. Typical PNA structures are shown in Nielsen et al., Science, Vol. 254:1497-1500 (1991) and Egholm et al. Nature,, Vol. 365:566-568 (1993) which are incorporated herein

by reference and attached hereto (Exhibits 7 and 8).

The concept and implementation of a primer having a 5' end beyond the 7th, 8th or 9th nucleotide from the 3' end to optimize or increase the efficiency or functions of the 3' end is entirely novel, as far as could be determined. It is virtually impossible to describe all the means to accomplish this or an equivalent objective or result. It is not to be excluded that others skilled in the art might benefit from the teaching herein and apply it to their own purposes. Accordingly, it is contemplated by the inventors and it is their intention that substantially equivalent means performing in substantially in the same manner and accomplishing substantially the same or better result be considered within the scope of the invention though such may not fall within the literal wording of the claims.

As taught by the invention, the number of necessary oligonucleotides for fingerprint visualization has been determined to be 3. When a primer having an "optimizer" like a "hairpin" at its 5' end, it is not inconceivable however, that this number may be further reduced to its logical limit of one nucleotide. Moreover, one or more of the nucleotides of the 3 first nucleotides of the 3' end could be replaced by a degenerate base.

Further, it has been observed that the "hairpin" recognizes the template strands. It is therefore feasible to cause amplification without a core of nucleotides, thus using the hairpin

as the primer.

The invention further provides means and methods for significantly further increasing the ability to detect polymorphisms. The method comprises digestion of a nucleic acid template with restriction endonucleases prior to amplification. This coupling of endonuclease cleavage and amplification of arbitrary stretches of DNA directed by short oligonucleotide primers allows ready distinction of closely related fungal and bacterial isolates, plant cultivars and eukaryotic organisms. MAAP analysis of cleaved template DNA identified molecular markers linked to a developmental locus of soybean (Glycine max L.). EMS-induced supernodulating near-isogenic lines altered in the nts locus that controls nodule formation could be distinguished from each other and from their parent cultivar by amplification of template pre-digested with 2-3 restriction enzymes. A total of 42 DNA polymorphisms were detected using only 19 octamer primers. In the absence of digestion, 25 primers failed to differentiate these soybean genotypes. Several polymorphic products co-segregated tightly with the nts locus in F_2 families from crosses between the allelic mutants nts382 and nts1007 and the ancestral *G. soja* Sieb. & Succ. PI468.397.

If desired, the pre-digestion may be followed by a post-digestion of the products of amplification with endonucleases.

Any primer may be used in this embodiment of the

invention. The primers herein discussed may be particularly useful. Mixtures of primers are suitable. Amplification was carried out with pentamers, heptamers or octamers. Longer oligonucleotide primers can be used also. The predigestion is preferably carried out to completion.

In another aspect of the invention where restriction nuclease digest genomic DNA, the DNA can be of animal, plant or human sources, as in the other embodiments of the invention. The number of restriction fragments with which the primers will be made to react depends on the size of the genome and the frequency of occurrence of the target site of the restriction endonuclease in the genome, which in turn is primarily determined by the number of nucleotides in the target site. The number of nucleotides in the target sites of commonly used restriction endonucleases ranges from 4 to 8. The genome sizes of organisms vary widely from a few million base pairs in the case of microorganisms to several billion base pairs for animals and plants. Hence, the number of restriction fragments obtained after cleaving genomic DNA molecules with a restriction enzyme can vary from a few hundred to several million.

Any restriction endonuclease which recognizes a specific base sequence in a double stranded DNA and will cleave both strands of the DNA molecule at every target site can be used. Both blunt-end or staggered cutting endonucleases were used. The DNA sequence

need not be a known sequence.

Typical useful endonucleases are the following: HaeIII, Sau3A, BamHI and MspI. Other restriction endonucleases are of course available and may be selected from Current Protocols in Molecular Biology, Vol. 1, ed. Ausubel et al., Section 3.1.6 through 3.1.20 which is incorporated herein by reference.

It is believed that cleavage at or near the restriction sites may make amplicons more readily available that are normally shielded and not available for replication.

For further description, see Molecular General Genetics, Vol. 241:64-67 (1993) incorporated herein by reference (Exhibit 9) and Applying DNA with Arbitrary Oligonucleotide Primers in Review, cited also and incorporated by reference and attached hereto.

DAF has been shown to be very useful to distinguish amongst closely related genotypes of prokaryotic and eukaryotic organisms. There are situations were genetic improvements in certain plant strains and their respective contributions are particularly difficult to distinguish one from another and from the original strain.

The spectrum of nucleic acids characteristic of a particular nucleic acid genome generated in accordance with the invention, are useful per se not only for visualizing as described herein but also a starting nucleic acids fragments for further uses. Such pool or library of nucleic acid products can be

obtained directly for the application of the process of the invention or by further treatment thereof, such by post-restriction endonuclease processing.

The application of the fingerprinting of the invention to mixed genomes is of great interest. Primers can be tailed to amplify preferentially and fingerprint a target genome in a mixture of their DNA fragments. An illustration of that situation is in nitrogen fixation. The *Azolla-Anabaena* symbiosis has been used for centuries as a nitrogen biofertilizer in rice paddies. Genetic improvement of the symbiosis has been limited by the difficulty in identifying *Azolla-Anabaena* accessions and *Anabaena* strains.

In this development, there were mixtures of DNA extracts from both prokaryotic *Anabaena* DNA and eukaryotic *Azolla* DNA. DAF could distinguish and positively identify accessions of *Azolla-Anabaena* with DNA extracted from intact symbiosis. For a detailed description see Plant Molecular Biology, Vol. 21:363-373 (1993) (Exhibit 10), which is incorporated herein by reference and attached hereto. Thus, there are circumstances in which it is helpful to use a complex mixture of nucleic acid, e.g. DNA fragments for amplification and profiling.

In accordance with the invention, multiple arbitrary primers can be used to generate fingerprints of prokaryotic and eukaryotic nucleic acids. Preferably the primers are of the same nucleotide length. Amplification products obtained using for

instance two primers, produced a fingerprint which was not merely the result of adding amplification products obtained separately with each individual primer. Certain bands disappeared while other new ones were generated and few bands were shared. Each primer amplifies discrete and limited portions of a genome, producing a characteristic set of amplification products. Where a multiplicity of primers is used, new products arise from the overlap of the extension products initiated by each primer while others disappear. Alteratively, competition for annealing sites during amplification could result in a generation of new fingerprint patterns. The technique of using several primers has been nick-named "multiplex".

Furthermore, for some genomes which are difficult to separate, multiplex DAF is another approach for generating DNA fingerprints. Pooling of several primers can also increase the chances of finding polymorphic DNA. If there are difficulties to reveal polymorphisms between a set of cultivars, indication is that a subset of the primers in the mix could reveal these polymorphisms individually, so that multiplex DAF would provide an initial use for screening for closely relates species.

For RNA amplification using arbitrary primers (nick-named this procedure cDAF for complementary DNA amplification fingerprinting) clear fingerprints were also produced. In all cases the material was RNA extracted from roots of soybean cv. Bragg from the region of emergent root hairs. Primers used were

CGCGGCCA, CGCGGCCA and TTTTTT (referred to as T_6), GCGC, GCGC and T_6, CCTGT, CCTGT and T_6, AATGC, and finally AATGC and T_6. All combinations produced fingerprints. In the studies, 0.5 μg/μl RNA was reverse transcribed with 20 U MoMuLV reverse transcriptase in the presence of 1 μg total primers, 2 U RN Aasin and appropriate buffer containing nucleotides and magnesium chloride, by incubating the mixture at 23°C for 10 min followed by an incubation at 42°C for 30 min. The resulting reaction was treated as template for DAF analysis with the same primers used for transcription.

Multiple primers have been used in conjunction with various embodiments of the DAF invention. DNA amplification of turfgrass DNA using multiple primers was used to enhance understanding of genome divergents, cultivar identity and genetic mapping of relative adaptive gene loci. See Golf Course Management, Vol. 61:80-86 (1993) which is incorporated herein by reference and attached (Exhibit 11). Multiple primers used in these investigations were nucleotides GTATCGGC+GACGTAGG. A DNA polymerase used was the truncated derivative, the so-called Stoffel fragment of AmpliTaq (Perkin-Elmer/Cetus).

Fingerprinting with mixtures of primers of sequences CGCGGCCA and GCTGGTGG and of CGCGGCCA, GCTGGTGG and AATGGAGC or Staphylococcus aureus FDA 574 revealed complex banding patterns. See Biotechnology, Vol. 9:553-557 (1991) cited above.

Other mixtures of primers ranging from 5 to 15 are useful

in the practice of the invention. There may be used 2 or more primers.

As known, most DNA polymerases perform primer extension reactions of the nucleic acid substrate consisting of a primer hybridizing to a template strand such that the 3' end of the primer is recessed relative to the 5' end of the template strand. The template strand is either a DNA or RNA. A DNA polymerase is used to extend the primer. For RNA templates, reverse transcriptase is an example of a nucleic acid polymerase that may be used. The choice of nucleic acid polymerase used in the extension reaction depends on the nature of the template.

It has been found in accordance with the invention that the truncated derivative, the so called Stoffel fragment of a DNA polymerase from <u>Thermos aquaticus</u> (AmpliTaq) is particularly useful. The Stoffel fragment is a highly thermostable, recombinant DNA polymerase, lacking the 289 N-terminal amino acids. It has a broad magnesium optimum, increased thermostability and no associated 3'-5' or 5'-3' exonuclease activity. The enzyme is described in a pamphlet from Perkins-Elmer/Cetus. The enzyme gave improved performance in DAF reactions and particularly is more efficient in amplification of short products and thus able to produce more informative fingerprints than other DNA polymerases. Preferably between about 0.2 and 0.4 units/μl of the Stoffel fragment produced clear and consistent results, whereas only 0.075

and 0.1 units/μl of AmpliTaq was suitable. Generally, the Stoffel fragment produced a broader distribution of amplification products each in greater amounts. This was especially evident with smaller products (less than 300 bp). Although both polymerases produced consistent fingerprints with 4 mM magnesium, the Stoffel enzyme was inhibited at a concentration higher than about 8 mM whereas the Stoffel fragment can tolerate concentration of magnesium up to about 12 mM.

Other DNA polymerase, preferably heat-stable which are at least substantially equivalent may be used like other N-terminally truncated *Thermus aquaticus* (Taq) DNA polymerase I. the polymerase named KlenTaq I and KlenTaq LA are quite suitable for that purpose. When KlenTaq I is substituted in the above described studies for the Stoffel fragment comparable results were obtained.

DNA from soybean cyst nematode (*Heterodera glycines*), and soybean DNA were amplified with primers GTAACGGCC and CCGAGCTG with KlenTaq I and KlenTaq LA.

In accordance with the invention, it has also been found that the annealing temperature in the cycling during the amplification reaction may be carried out within temperature ranges significantly above those commonly used heretofore, generally about 30°C - 35°C, the typical cycling parameters for PCR and RAPD.

It is noteworthy that primers of 7-15 nucleotides provided readable and meaningful profiles in the temperature range

of about 15°C through 75°C. The heptamers produced DNA amplification in the range of 15°C through 60°C, preferably 45°C through 60°C. A pentamer still produces DAF products at about 55°C. Octamers provide meaningful profiles in the range of about 15° to 65°C. It appears that DAF products are quite tolerant of annealing, extension, and denaturing times as opposed to other MAAP techniques.

Generally, amplification is carried through from 2 to as many cycles as is optimum to result in adequate number of amplified products for visualization such as up to 35 cycles., i.e. of 30 seconds at 96°C, 30 seconds at the appropriate annealing temperature and 30 seconds at 72°C in a recirculating air-thermocycler (Bios, New Haven, CT.).

Generally, especially for DAF products of less than about 500bp, a two step cycling between the melting temperature (96°C) and a non-stringent prime annealing temperature (in the ranges described) is sufficient for amplification.

An extension step is not necessary. Consistent products were obtained after 30-35 cycles and cycle numbers as high as 50 can be used without effecting fingerprint quality.

In accordance with the invention it has also been found that it is advantageous to use primer concentrations, than has been used heretofore, particularly relative to the amount of nucleic acid template. Whereas the prior art MAAP techniques other than

DAF generally used DNA template concentration in excess of primer concentration, the DAF method provides concentration of primers in excess of DNA template.

to use about 3 μM primer concentrations for 7mer to 15mer primers, and about 10-30 μM concentrations for shorter primers (5mer and 6mer). For primers longer than 15mer primer concentration less than 3 μM such as about 2μM or less, gives satisfactory results. Amplification with pentamers are often performed with about 30 μM. Template concentrations generally used range from 0.1 ng/μl to 100 ng/μl. A minimum of 1 ng/μl template is sufficient to produce reliable fingerprints of low complexity genomes like prokaryotes or bacteriophage. A practical upper limit of template concentrations of 1 ng/μl appear quite satisfactory at this time.

A 3 μM solution of primer ranges from approximately 5 to 1 ng/μl for 5mer to 10mer primers, respectively.

With certain templates of high complexity eukaryotes (plants, animals, mammals, humans, etc.), there can be used as low as about 1 pg of total DNA (1 μg primer) defining a 1,000,000 ratio upper level. When using 0.2 μg total primer in the reaction, the upper level is preferably a 200,000 ratio primer/template.

Typically, to amplify bacteria with a 5mer (5 ng/μl primer to 1 ng/μl template), the preferred ratio is about 5, which in some circumstances can be brought down to about 2. With an

octamer(8 ng/μl primer to 1 ng/μl template), the preferred ratio is about 8. To amplify complex genomes like soybean and mammals with a 5mer (5 ng/μl primer to 0.1 ng/μl template) the ratio is about 50. For primers of 5 to 8 or 9 nucleotides, it is preferrred that the ratio be about 5 to 80 or 90, respectively. For an octamer, the ratio is about 80. A preferred practical range of minimum and maximum range of primer is preferably from about 2 to 1,000,000 relative to the template, preferably about 5 to about 200,000.

These parameters are provided as a guideline to one skilled in the art. It is contemplated that if one skilled in the art would work outside of these guidelines (ratios, limits, etc.), perhaps making other adjustments to other operative parameters, he would still be within the spirit if not the letter of the invention and its teaching.

In accordance with the invention it has been noted above that the various improvements in methodology and new products herein disclosed can be used individually in conjunction with what is called the DAF method or in general with MAAP methodologies, or in any combination thereof. For instance, the feature of the invention using an arbitrary mini-hairpin oligonucleotide primer for nucleic acid amplification fingerprinting with only 3 nucleotides can be applied to any existing MAAP techniques or others which will be hereinafter discovered. The mini-hairpin

primers disclosed herein are expected to be useful in the amplification of any nucleic acid using polymerization with the described and other DNA or RNA polymerases. Equivalent primers having a 5' end which enhances the amplification of amplicons can also be used in other MAAP techniques. In accordance with the teaching of the invention reamplification of the products of the reaction can be performed whether or not such above-described primers were used. Likewise, the predigestion with endonuclease restriction enzymes can be applied to the heretofore DAF process alone or together with any of the other improvements disclosed herein. Likewise, the predigestion of the template by endonuclease restriction enzymes can be carried out on products traditionally used for other than DAF or MAAP processes, including the PCR methods. See Mullis et al., U.S. Patent No. 4,683,195 and Mullis et al., U.S. Patent No. 4,683,202 which disclosed polymerase chain reactions which can be used to amplify any specific segment of nucleic acid.

Comparison of the products in accordance with the invention can be accomplished by a variety of techniques known to those skilled in the art. Inspection of the electrophoresis gel of the products reveals polymorphisms that affect the size and quantity of the amplified segment and polymorphisms that determine what segment is amplified. A preferred method for size fractionation is electrophoresis through a polyacrylamide gel or

agarose gel matrix. A more preferred method of visualizing the products of the invention is by the method disclosed in the second parent patent application and in co-pending application Serial No. ________________. Other literature references that may be helpful in the visualization of the products of the invention are found in the various articles above referred to and which are hereby incorporated by reference.

Various text books and laboratory manuals which may be useful to one skilled in the art if desired include the following: Current Protocols, Vols. 1 and 2, ed. Ausubel et al..

As will be apparent to those skilled in the art, in light of the foregoing disclosure, many modifications, alternations and substitutions are possible in the practice of this invention without departing from the spirit or the scope thereof. The resulting profiles of multiple primers are highly informative and may be preferred to the use of one primer in certain circumstances.

We claim:

1. A method for profiling a sample of nucleic acids of unknown sequence and determining whether the nucleic acid sequence is the same or different from another nucleic acid sequence, which method comprises:

a) treating at least one strand of nucleic acids with an oligonucleotide primer which comprises 5 to 25 nucleotides, and allowing the primer to anneal to multiple arbitrary sites on each strand of the nucleic acids template, each site being substantially complementary to the nucleotides of which the primer is constituted, thus forming a multiplicity of a first set of primed templates,

b) treating said primed templates with a nucleic acid polymerase, thereby generating a multiplicity of extension strands, the extension strands comprising the primer in combination with a sequence of nucleotides that is substantially complementary to the templates, and extending along the template strands, terminating either at the 5' end of the template strands or at the nucleotide prior to the next primed site on the templates, the number of extension strands thereby corresponding substantially to the number of primed sites,

c) denaturing the extension strands from the template strands so that each of the newly synthesized extension

strands and the first set of template strands function as templates, wherein the concentration of primer to template is in the range of about 2 to about 1,000,000.

d) repeating steps (a), (b) and (c) thereby generating a spectrum of nucleic acid fragments which are characteristic and unique for the unknown sequence of nucleic acids,

e) separating said spectrum of fragments, and

f) determining the characteristic profile of fragments associated with said sequence of nucleic acids, the profile being a highly complex multibanded profile.

2. The method of claim 1 wherein the ratio is in the range of about 5 to about 200,000.

3. The method of claim 2 wherein the ratio is in the range of about 5 to about 80, when the primer is of 5 to 8 nucleotides.

4. The method of claim 3 wherein the ratio is in about 8, when the primer has 8 nucleotides.

5. An oligodeoxyribonucleotide primer which comprises at its 5' end a hairpin structure and at its 3' end, a nucleotide

sequence.

6. The primer of claim 5 wherein the nucleotide sequence has at least 3 nucleotides.

7. The primer of claim 6 wherein the nucleotide sequence has 3 to 8 nucleotides.

8. The primer of claim 6 wherein the hairpin is constituted of a stem of at least two complementary bases and a single stranded loop.

9. The primer of claim 6 wherein the hairpin has from 2 to 12 nucleotides.

10. The primer of claim 7 wherein the nucleotide sequence has 3 nucleotides.

11. A combination of 64 different primers of claim 15, which constitutes the total of all combinations in a sequence of 3 nucleotides containing any of the known 4 bases of DNA, the nucleotide hairpin sequence being identical.

12. A method for profiling a sample of nucleic acids of

unknown sequence and determining whether the nucleic acid sequence is the same or different from another nucleic acid sequence, which method comprises:

a) treating at least one strand of nucleic acids with a hairpin oligonucleotide primer and allowing the primer to anneal to multiple arbitrary sites on each strand of the nucleic acids template, each site being substantially complementary to the nucleotides of which the primer is constituted, thus forming a multiplicity of a first set of primed templates,

b) treating said primed templates with a nucleic acid polymerase, thereby generating a multiplicity of extension strands, the extension strands comprising the primer in combination with a sequence of nucleotides that is substantially complementary to the templates, and extending along the template strands, terminating either at the 5' end of the template strands or at the nucleotide prior to the next primed site on the templates, the number of extension strands thereby corresponding substantially to the number of primed sites,

c) denaturing the extension strands from the template strands so that each of the newly synthesized extension strands and the first set of template strands function as templates,

d) repeating steps (a), (b) and (c) thereby generating a spectrum of nucleic acid fragments which are

characteristic and unique for the unknown sequence of nucleic acids,

e) separating said spectrum of fragments, and

f) determining the characteristic profile of fragments associated with said sequence of nucleic acids, the profile being a highly complex multibanded profile, the primer being a hairpin primer.

13. The method of claim 12 wherein the primer comprises at its 5' end a hairpin structure and at its 3' end, a nucleotide sequence.

14. The method of claim 13 is constituted of the hairpin has from 2 to 12 nucleotides.

15. The method of claim 13 wherein the nucleotide sequence has 3 to 8 nucleotides.

16. The method of claim 15 wherein the nucleotide sequence has 3 nucleotides.

17. A method for profiling a sample of nucleic acids of unknown sequence and determining whether the nucleic acid sequence is the same or different from another nucleic acid

sequence, which method comprises:

a) treating at least one strand of nucleic acids with an oligonucleotide primer of 5 to 15 nucleotides, and allowing the primer to anneal to multiple arbitrary sites on each strand of the nucleic acids template, each site being substantially complementary to the nucleotides of which the primer is constituted, thus forming a multiplicity of a first set of primed templates,

b) treating said primed templates with a nucleic acid polymerase, thereby generating a multiplicity of extension strands, the extension strands comprising the primer in combination with a sequence of nucleotides that is substantially complementary to the templates, and extending along the template strands, terminating either at the 5' end of the template strands or at the nucleotide prior to the next primed site on the templates, the number of extension strands thereby corresponding substantially to the number of primed sites,

c) denaturing the extension strands from the template strands so that each of the newly synthesized extension strands and the first set of template strands function as templates,

d) repeating steps (a), (b) and (c) thereby generating a spectrum of nucleic acid fragments which are characteristic and unique for the unknown sequence of nucleic acids,

e) separating said spectrum of fragments, and

f) determining the characteristic profile of fragments associated with said sequence of nucleic acids, the profile being a highly complex multibanded profile,

the primer having a 3' end of 3 nucleotides and a modified 5' end which functions to optimize the role of the core domain of the primer in searching for and annealing to target sites, thereby maximizing the information content of the spectrum of generated fragments

18. The method of claim 17 wherein the modified 5' end is a polyamide nucleic acid DNA hybrid.

19. The method of claim 18 wherein the modified 5' end is a fluophore radical.

20. The method of claim 1 wherein the nucleic acids in the sample are DNA sequences which have been cleaved with restriction endonuclease to yield restriction fragments.

21. The method of claim 1 wherein the nucleic acid polymerase is a truncated fragment of AmpliTaq.

22. The method of claim 21 wherein the polymerase is KlenTaq I.

23. The method of claim 1 which comprises after step (d) and prior to steps (e) and (f), a post-treatment for reamplification of the amplified nucleic acid fragments which comprises separating a first spectrum of the fragments, repeating steps (a), (b) and (c) on said spectrum with a longer primer than used in step (a) and repeating steps (a), (b) and (c) on said first spectrum, thereby generating a second set of spectrum of fragments, (e) separating said second set of fragments and (d) determining the characteristic profile of fragments associated with said sequence of nucleic acids, the profile being different and more characteristic that would have been obtained from a profile of the first spectrum of fragments without the reamplification by post-treatment.

24. The method of post-treatment of claim 23 which comprises performing said method on an isolated and purified fragment from the first spectrum of fragments.

25. The method of claim 1 wherein the primer has from 7 to 15 nucleotides which comprises carrying out step (b), the annealing step at a temperature in the range of about 15 to about

75^0C.

26. The method of claim 25 wherein the primer has from 7 to 10 nucleotides and the annealing step is performed at a temperature in the range of about 15 to about 65^0C.

27. The method of claim 26 wherein the primer has 8 nucleotides and wherein the temperature is in the range of about 45 to about 65^0C.

28. The method of claim 1 wherein the treatment of the nucleic acid is performed concurrently with two primers, thereby generating a profile more characteristic than would have been generated by one of the primer.

29. The method of claim 28 wherein the treatment is performed with two primers which are of the same nucleotide length.

30. The method of claim 29 wherein the nucleic acid polymerase is an N-terminally truncated fragment of Taq DNA polymerase I.

31. The method of claim 39 wherein the method is carried out on nucleic acid samples of closely related plant genomes.

32. The nucleic acid mixture obtained from the process of claim 1.

33. The nucleic acid mixture obtained from the process of claim 20.

34. The nucleic acid mixture obtained from the process of claim 33.

35. The method of claim 1 which comprises visualizing the spectrum of nucleic acids on a dried and developed polyacrylamide gel.

36. The developed and dried gel which is the product of the method of claim 34, which has a permanent record of the original nucleic acid fragments.

37. The gel of claim 35 which is a backed supported gel.

AMENDED CLAIMS

[received by the International Bureau on 08 May 1995 (08.05.95); original claims 5-10 and 13 cancelled; original claims 1-4, 11,12,14,14-17,32-34,36 and 37 amended; new claims 38-45 added; remaining claims unchanged (8 pages)]

1. A method for generating a pattern characteristic of an unspecified nucleic acid sequence in a sample, which sequence is a template, which method comprises:

a) treating at least one strand of nucleic acids with an oligonucleotide primer which comprises 5 to 25 nucleotides, and allowing the primer to anneal to multiple arbitrary sites on each strand of the nucleic acids template, each site being substantially complementary to the nucleotides of which the primer is constituted, thus forming a multiplicity of a first set of primed templates,

b) treating said primed templates with a nucleic acid polymerase, thereby generating a multiplicity of extension strands, the extension strands comprising the primer in combination with a sequence of nucleotides that is substantially complementary to the templates, and extending along the template strands, terminating either at the 5' end of the template strands or at the nucleotide prior to the next primed site on the templates, the number of extension strands thereby corresponding substantially to the number of primed sites,

c) denaturing the extension strands from the template strands so that each of the newly synthesized extension strands and the first set of template strands function as templates, wherein the concentration of primer is in excess to that of template.

d) repeating steps (a), (b) and (c) thereby generating a spectrum of nucleic acid fragments which are characteristic and unique for the unspecified sequence of nucleic acids,

e) separating said spectrum of fragments, and

2. The method of claim 38 wherein the ratio of primer to template is in the range of about 5 to about 200,000.

3. The method of claim 2 wherein the ratio of primer to template is in the range of about 5 to about 80, when the primer is of 5 to 8 nucleotides.

4. The method of claim 3 wherein the ratio of primer to template is about 8, when the primer has 8 nucleotides.

11. A combination of 64 different oligonucleotide primers of claim 40, wherein the 3' single stranded end extending from the double stranded stem comprises 3 nucleotides, wherein the combination constitutes the total of all combinations in a sequence of 3 nucleotides containing any of the known 4 bases of DNA, and wherein the portion of the 64 oligonucleotide primers other than the 3' single stranded end extending from the double stranded stem contain identical sequences in each of the primers.

12. A method for generating a pattern characteristic of an unspecified nucleic acid sequence in a sample, which sequence is a template, which method comprises:

a) treating at least one strand of nucleic acids with the oligonucleotide primer of claim 40 and allowing the primer to anneal to multiple arbitrary sites on each strand of the nucleic acids template, each site being substantially complementary to the nucleotides of which the primer is constituted, thus forming a multiplicity of a first set of primed templates,

b) treating said primed templates with a nucleic acid polymerase, thereby generating a multiplicity

of extension strands, the extension strands comprising the primer in combination with a sequence of nucleotides that is substantially complementary to the templates, and extending along the template strands, terminating either at the 5' end of the template strands or at the nucleotide prior to the next primed site on the templates, the number of extension strands thereby corresponding substantially to the number of primed sites,

c) denaturing the extension strands from the template strands so that each of the newly synthesized extension strands and the first set of template strands function as templates,

d) repeating steps (a), (b) and (c) thereby generating a spectrum of nucleic acid fragments which are characteristic and unique for the unspecified sequence of nucleic acids,

e) separating said spectrum of fragments, and

f) determining the characteristic profile of fragments associated with said sequence of nucleic acids.

14. The method of claim 12 wherein the loop has 3 or 4 nucleotides.

15. The method of claim 12 wherein the single strand has 3 to 8 nucleotides.

16. The method of claim 15 wherein the single strand has 3 nucleotides.

17. A method for generating a pattern characteristic of an unspecified nucleic acid sequence in a sample, which sequence is a template, which method comprises:

a) treating at least one strand of nucleic acids with an oligonucleotide primer of 5 to 15 nucleotides, and allowing the primer to anneal to multiple arbitrary sites on each strand of the nucleic acids template, each site being substantially complementary to the nucleotides of which the primer is constituted, thus forming a multiplicity of a first set of primed templates,

b) treating said primed templates with a nucleic acid polymerase, thereby generating a multiplicity of extension strands, the extension strands comprising the primer in combination with a sequence of nucleotides that is substantially complementary to the templates, and extending along the template strands, terminating either at the 5' end of the template strands or at the nucleotide prior to the next primed site on the templates, the number of extension strands thereby corresponding substantially to the number of primed sites,

c) denaturing the extension strands from the template strands so that each of the newly synthesized extension strands and the first set of template strands function as templates,

d) repeating steps (a), (b) and (c) thereby generating a spectrum of nucleic acid fragments which are characteristic and unique for the unspecified sequence of nucleic acids,

e) separating said spectrum of fragments, and

f) determining the characteristic profile of fragments associated with said sequence of nucleic acids,

the primer having a 3' end of 3 nucleotides and a modified 5' end which functions to optimize the role of the core domain of the primer in searching for and annealing to target sites, thereby maximizing the information content of the spectrum of generated fragments

18. The method of claim 17 wherein the modified 5' end is a polyamide nucleic acid DNA hybrid.

19. The method of claim 18 wherein the modified 5' end is a fluophore radical.

20. The method of claim 1 wherein the nucleic acids in the sample are DNA sequences which have been cleaved with restriction endonuclease to yield restriction fragments.

21. The method of claim 1 wherein the nucleic acid polymerase is a truncated fragment of AmpliTaq.

22. The method of claim 21 wherein the polymerase is KlenTaq I.

23. The method of claim 1 which comprises after step (d) and prior to steps (e) and (f), a post-treatment for reamplification of the amplified nucleic acid fragments which comprises separating a first spectrum of the fragments, repeating steps (a), (b) and (c) on said spectrum with a longer primer than used in step (a) and repeating steps (a), (b) and (c) on said first spectrum, thereby generating a second set of spectrum of fragments, (e) separating said second set of fragments and (d) determining the characteristic profile of fragments associated with said sequence of nucleic acids, the profile being different and more characteristic that would have been obtained from a profile of the first spectrum of fragments without the reamplification by post-treatment.

24. The method of post-treatment of claim 23 which comprises performing said method on an isolated and purified fragment from the first spectrum of fragments.

25. The method of claim 1 wherein the primer has from 7 to 15 nucleotides which comprises carrying out step (b), the annealing step at a temperature in the range of about 15 to about 75°C.

26. The method of claim 25 wherein the primer has from 7 to 10 nucleotides and the annealing step is performed at a temperature in the range of about 15 to about 65°C.

27. The method of claim 26 wherein the primer has 8 nucleotides and wherein the temperature is in the range of about 45 to about 65°C.

28. The method of claim 1 wherein the treatment of the nucleic acid is performed concurrently with two primers, thereby generating a profile more characteristic than would have been generated by one of the primer.

29. The method of claim 28 wherein the treatment is performed with two primers which are of the same nucleotide length.

30. The method of claim 29 wherein the nucleic acid polymerase is an N-terminally truncated fragment of Taq DNA polymerase I.

31. The method of claim 39 wherein the method is carried out on nucleic acid samples of closely related plant genomes.

32. The separated fragments obtained from the method of claim 1.

33. The separated fragments obtained from the method of claim 12.

34. The separated fragments obtained from the method of claim 17.

35. The method of claim 1 which comprises visualizing the spectrum of nucleic acids on a dried and developed polyacrylamide gel.

36. The developed and dried gel which is the product of the method of claim 35, which has a permanent record of the original nucleic acid fragments.

37. The gel of claim 36 which is a backed supported gel.

38. The method of claim 1 wherein the ratio of primer to template is in the range of about 2 to about 1,000,000.

39. The method of claim 1 wherein the oligonucleotide primer has a 3' single stranded end and a 5' end and which primer comprises a double stranded stem of at least 2 complementary annealed base pairs, the double stranded stem extending to the 5' end of the primer and the other end of the stem extending as the single strand of at least 2 bases to the 3' end of the primer, and intermediate to the 5' and 3' ends of the stem, a single stranded loop of at least 3 bases.

40. An oligonucleotide primer having a 3' single stranded end and a 5' end which primer comprises a double stranded stem of at least 2 complementary annealed base pairs, the double stranded stem extending to the 5' end of the primer and the other end of the stem extending as the single strand of at least 2 bases to the 3' end of the primer, and intermediate to the 5' and 3' ends of the stem, a single stranded loop of at least 3 bases.

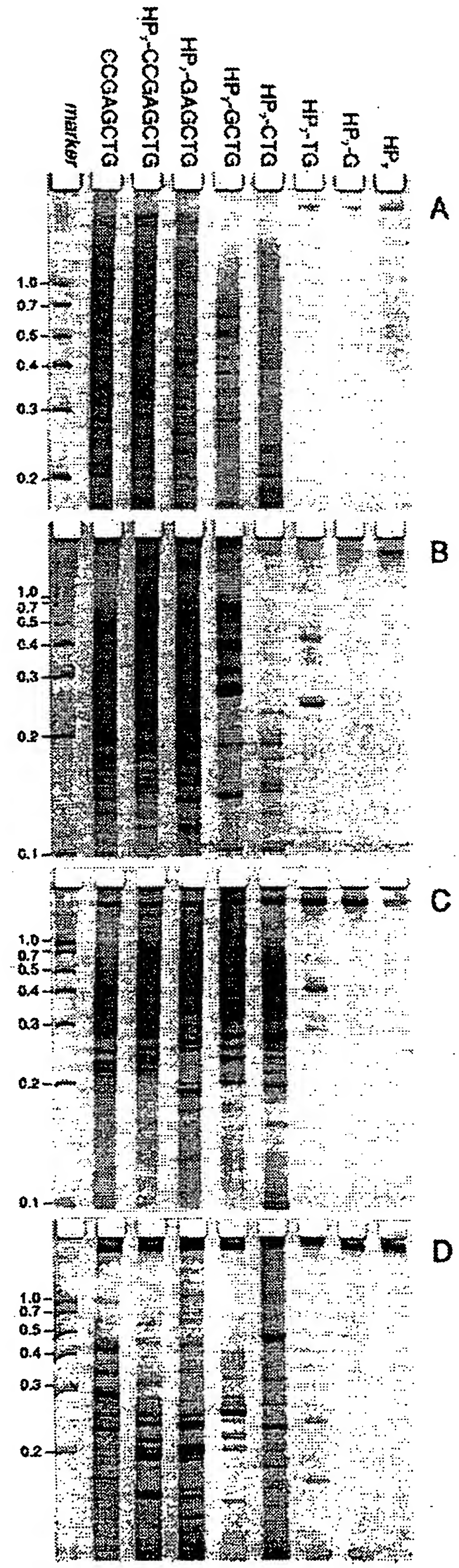
41. The oligonucleotide primer of claim 40 wherein the loop has 3 bases.

42. The oligonucleotide primer of claim 40 wherein the loop has 4 bases.

43. The oligonucleotide primer of claim 40 wherein the length of the single strand is of 3, 4, 5, 6, or 8 nucleotides.

44. The oligonucleotide primer of claim 40 which is a deoxyribonucleotide sequence.

45. The oligonucleotide primer of claim 40 which is a ribonucleotide sequence.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B
C
D
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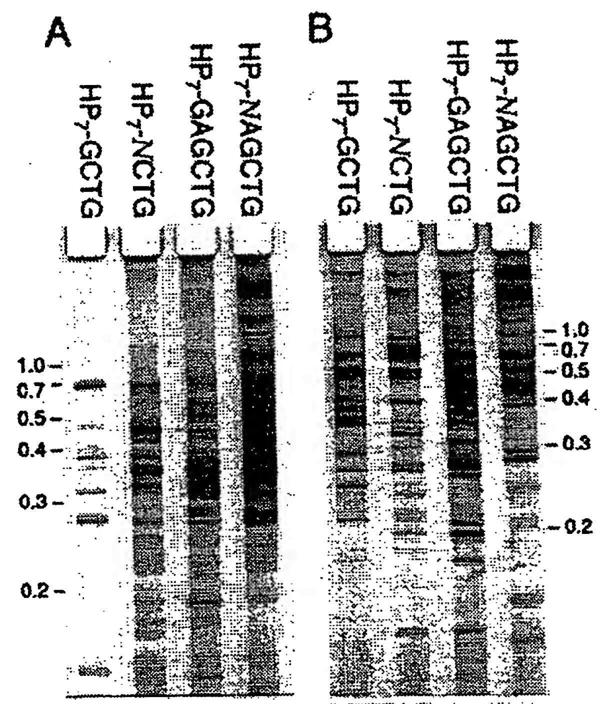
0.2
0.1

FIG. 1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B
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HPγ-NAGCTG
1.0
0.7
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0.3
0.2
1.0
0.7
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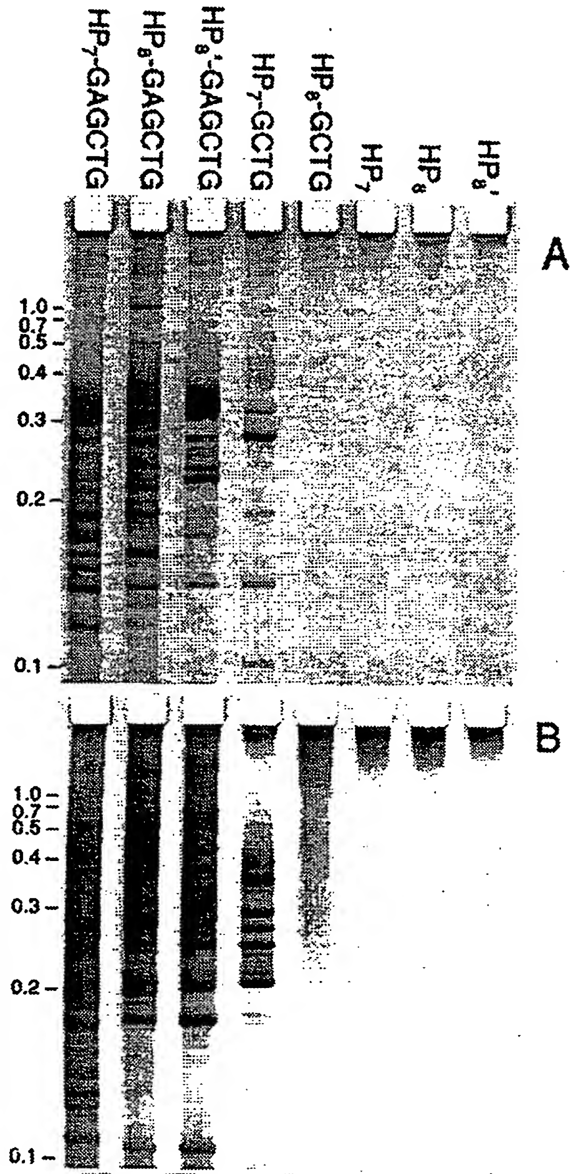
0.3
0.2

FIG. 2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HP8'-GAGCTG
HP7-GCTG
HP8-GCTG
HP7
HP8
HP8'
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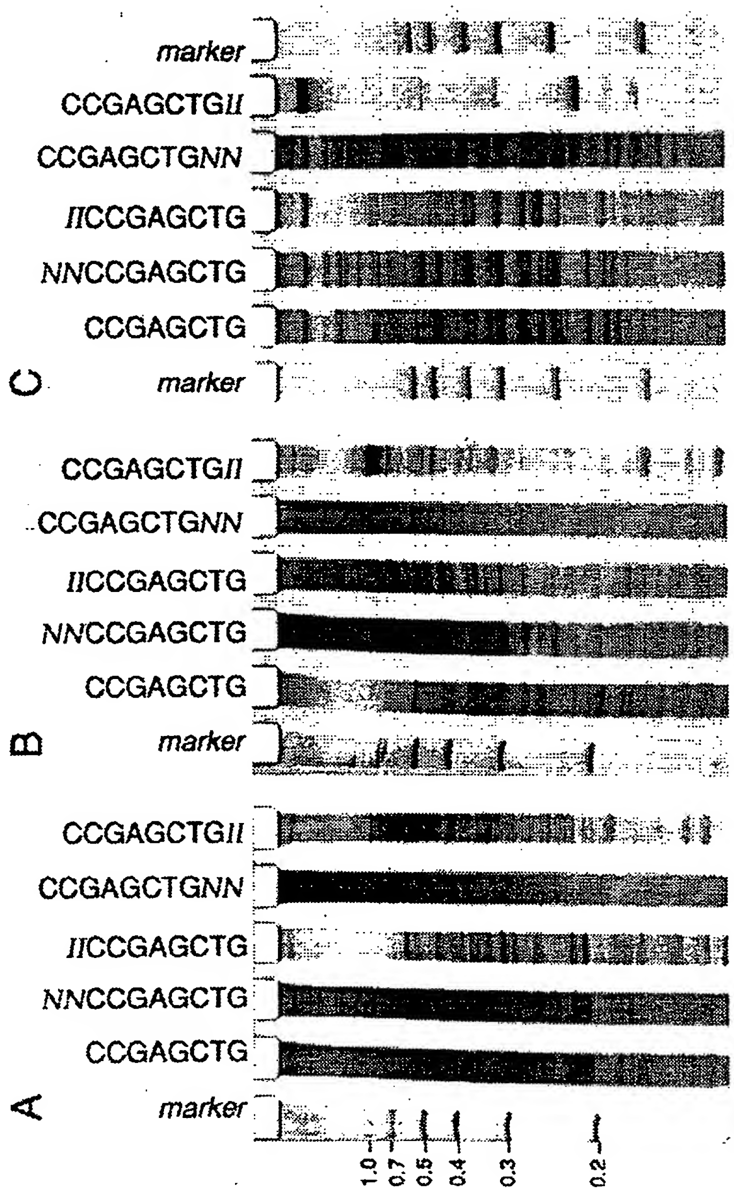
0.4
0.3
0.2
0.1

FIG. 3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NNCCGAGCTG
CCGAGCTG
B
marker
CCGAGCTGII
CCGAGCTGNN
IICCGAGCTG
NNCCGAGCTG
CCGAGCTG
A
marker
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0.7
0.5
0.4
0.3
0.2

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/11919

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR & GENERAL GENETICS, vol.241, no.1-2, October 1993, BERLIN, DE. pages 57 - 64 CAETANO-ANOLLES, G ET AL 'ENHANCED DETECTION OF POLYMORPHIC DNA BY MULIPLE ARBITRARY AMPLICON PROFILING OF ENDONUCLEASE-DIGESTED DNA' see the whole document ---	1-4, 20-37
X	MOLECULAR & GENERAL GENETICS, vol.235, no.2-3, October 1992, BERLIN, DE pages 157 - 165 CAETANO-ANOLLES, G ET AL 'PRIMER-TEMPLATE INTERACTIONS DURING DNA AMPLIFICATION PRINTING WITH SINGLE OLIGONUCLEOTIDES' see the whole document ---	1-4, 21-37
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1995	08.03.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 94/11919

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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